UV-VISIBLE METABOLOMICS APPROACH FOR THE DETERMINATION OF SELECTED ADULTERANTS IN CLAIMED PREMIXED COFFEE.

By

MUHAMMAD ZUL FAYYADH BIN AZIZO RAHMAN

Thesis submitted in partial fulfilment of the requirements for Master of Sciences

(Health Toxicology)

2015

ACKNOWLEDGEMENTS

Bismillahirrahmanirrahim,

Alhamdulillah. First of all, I am grateful to Allah for the strengths and His blessing in completing this thesis entitled "*UV-Vis metabolomics approach for the determination of selected adulterants in claimed premixed coffee*". I would like to express my deepest appreciation to Dr. Lim Vuanghao, for his supervision, constructive comments and constant support throughout the laboratory work and thesis writing that have contributed to the success of this research. Special appreciation also goes to my family for their abiding love and supports.

Deepest thanks and appreciation to all laboratory staffs especially those in Integrative Medicine cluster for their expertise guidance and moral support from the beginning till the end of this research. In addition, thanks to my friends for their advice and moral support.

Last but not least, my deepest gratitude to Advanced Medical and Dental Institute, Universiti Sains Malaysia (USM), the place where I conducted my research and for the sponsorship given to me.

_____

Muhammad Zul Fayyadh Bin Azizo Rahman

ABSTRACT

Adulterated premixed coffees have becoming an issue in Malaysia lately and has caught the eye of authorities due to death reports related to these products. The major cause of this issue reported that these premixed coffee passed the food inspection test and eventually released to the market for public. The coffee was claimed to be spiked with several sexual enhancers like sildenafil, tadalafil and vardenafill which are common drugs for treating erectile dysfunction. Metabolomics approach using UV-Vis spectroscopy was developed to detect the selected sexual enhancer drugs in the coffee commercial by employing SIMCA-P software version 13.0. Seven brands of coffee samples were purchased from local supermarket, 30 sachet each was tested totaling to 210 sample. Each sample were named H, J, G, W, N, T and K respectively. Three multivariate models were generated namely PCA, OPLS-DA and PLS-DA. OPLS-DA was chosen as the best model for overall results as it has minimal discriminate. Sildenafil, tadalafil and vardenafil were detected sample in H, vardenafil in brand J and none in sample G, W, N, T and K. The UV-Vis spectroscopy method had proven to be reliable and efficient in the determination of the selected drugs, as well as in saving time and cost.

ABSTRAK

Kopi pracampuran yang dicemari telah menjadi isu di Malaysia sejak kebelakangan ini dan telah menarik perhatian pihak berkuasa akan kerana laporan kematian yang berkaitan dengan produk-produk ini. Puncanya adalah kerana kopi ini melepasi pemeriksaan pemakanaan dan dipasarkan kepada awam secara berleluasa. Kopi ini telah didakwa bercampur dengan beberapa perangsang seks seperti sildenafil, tadalafil dan vardenafill adalah ubat yang sama untuk merawat mati pucuk. Kaedah metabolomics menggunakan UV-Vis spektroskopi diguna pakai untuk mengesan dadah yang disyaki dalam kopi dengan menggunakan perisian SIMCA-P versi 13.0. Tujuh jenama kopi diperoleh secara komersial, 30 bungkus setiap satu diuji yang berjumlah 210 sampel. Setiap jenama masing-masing dinamakan sebagai H, J, G, W, N, T dan K. Tiga model multivariat telah dihasilkan iaitu PCA, OPLS-DA dan PLS-DA. OPLS-DA diambil sebagai modal yang terbaik untuk hasil keseluruhan kerana ia mempunyai diskriminasi yang kurang. Sildenafil, tadalafil dan vardenafil hadir dikesan di dalam sample dalam H, vardenafil di dalam sample J dan tiada dalam sample G, W, N, T dan K. Kaedah UV-Vis spektroskopi telah terbukti akan keupayaan dan cekap dalam pengesanan dan juga dalam penjimatkan masa dan kos.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

cGMP	Cyclic guanosine monophosphate
PDE 5	Phosphodiesterase type 5
PDE 1	Phosphodiesterase type 1
PDE 6	Phosphodiesterase type 6
BPH	Benign prostatic hyperplasia
NAION	Nonarteritic anterior ischemic optic neuropathy
CYP3A4	Cytochrome p450 3A4
UV	Ultraviolet
λmax	Maximum Absorption
PCA	Principal component analysis
PC	Principal component
PLS	Partial least squares regression
PLS-DA	Partial least squares Discriminant Analysis
OPLS-DA	Orthogonal partial least squares Discriminant Analysis
MVDA	Multivariate Data Analysis
RSD	Residual standard deviation
VIP	Variable Influence on Projection
HPLC	High Performance Liquid Chromatography
NMR	Nuclear Magnetic Resonance

LIST OF SYMBOLS

ºC	Degree Celsius
%	Percent
λ	Lambda
X, Y and Z	Spatial coordinate
ml	Milliliter
mg	Milligram
nm	Nanometer
R^2	Goodness of fit
Q^2	Goodness of prediction
vs.	Versus
n^2	Square

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

Coffee gives the feeling of friendliness and daily stimulant for most people and working environment (Gracia *et al*., 2009). It is a standout amongst the most consumed refreshments on the planet (Gracia *et al*., 2009). Coffee is a special type of beverage made from coffee beans roasted to perfection. These bean comes from coffee berry's seed from the coffee plant. Equatorial regions of the world are the best spot for coffee to be cultivated especially in Southeast Asia, India, Africa, and America. Arabica and Robusta are the most common coffee selected by farmers to be grown as they are easy to be taken care of and are highly sought after. Once ripe, the berries are picked and only the seeds are taken under the process of drying. Coffee beans are very high in the market value and ranked top spot in agriculture trading around the world (Gracia *et al*. 2009). Once sold, the beans are roasted to different degrees, which give rise to different aromas, taste and flavour desired by the buyers.

Coffee is known to be mildly acidic with the pH value of 5-6. Unlike tea they also have caffeine which has a very positive and stimulating effect to humans booster. Coffee itself can also be used for other industries such as insect repellent, fertilizer, and medicine. However, coffee during the present days have been mixed with adulteration to enhance the coffee in different ways. "Enhancement Coffee" or "Herbal Coffee" is widely used by the people of South East Asia especially Malaysia. These coffees are in high demand due to the ability in enhancing their libido, vitality and overall health . The claimed coffee is also

consumed as beauty product amongst women. These products contain the mixture of either Tongkat Ali (*Eurycoma longifolia*), Kacip Fatimah (*Labisa Pumila*) and/or Ginseng (*Panax*). These mixed coffees posses good properties in boosting our immune system.

To avoid contaminated coffee from being price as the same as genuine coffee, the coffee quality is checked thoroughly before being released into the market and contaminated coffee will always be rejected (Prodolliet *et al*., 1995). Most coffee dealers in Malaysia abused the property of coffee by spiking adulterants (libido drug) into the original coffee. There were reports on health issues when consuming the coffee. This issue is rising since the production of illegal coffee in the black market which claims to have the same effect as 'Tongkat Ali", "Kacip Fatimah" and "Ginseng". In most cases, these illegal coffees were spiked with certain drug such as sildenafil, vardenafil, and tadalafil. These drugs were used as sexual enhancer in humans as they contain active pharmaceutical ingredients found in viagra (Vardi and Nini, 2007 and Bischoff, 2004)

Therefore the main objective of this research is to detect various types of adulterants in the premixed coffees which claimed to be sexual booster, wellness and vitality based on the information on the product packaging. The critical issue regarding these adulterated coffees has caught the attention of authority with reports on the drug content in the spiked coffee. Until recently, late last year the authorities have apprehended several companies selling contaminated coffee due to reported death cases in Malaysia and the products have been banned. The Health Ministry of Malaysia has been alerted on this issue due to the loose inspection of the permit approval system.

In this project, UV-Visible spectroscopy (Perkin Elmer, Waltham, Massachusetts, United States of America) and multivariate data analysis software, SIMCA-P version 13.0 (Umetrics, Umeå, Sweden) were employed to determine the presence of adulterant in 7 claimed coffee samples. Highly conjugated organic compounds which consist of sildenafil, tadalafil and vardenafil, which normally contain chromophores can be analyzed by the UV-Vis spectroscopy. Colors of the chemicals involved are directly perceived by the affected visible range by the absorption or reflectance. They undergo an electronic transition which mimics that of fluorescence directly which deals with transitions from the excited state to the ground state, while absorption is vice versa (Skoog *et al*., 2007). Chemometrics on the other hand, is the science which deal in extracting the information from chemical systems by data-driven means. This method is used to test whether it is viable to detect adulterants in premixed claimed coffee and to test the effectiveness of the method used.

1.1 Objectives

1) To determine the adulterants (sildenafil, tadalafil, and verdabafil) in coffee by using the metabolomics approach.

2) To demonstrate that UV-Vis method can be used as a rapid and effective method for the characterization of coffee.

1.2 Hypothesis

1) Adulterant drugs such as sildenafil , vardenafil and tadalafil are present in the claimed coffee.

2) Adulterant drugs are not present in the claimed coffee sold in the market.

CHAPTER 2

LITERATURE REVIEW

2.1 Coffee

The word coffee derived from many languages which dated as early as the 15th century where the Ottoman empire at that time called it as "kahve" (Gracia *et al*., 2009). Whereas the Arabic named was "qahwah" which came from the word "quwwa" meaning energy or power (Gracia *et al*., 2009). Most of the people where coffee was originated called it as dark wine and the exact language for it is hard to pin point to these day as nobody know where the word truly originated (Anonymous, 1999). The most credible evidence to date where coffee was first found was exactly in the 15th century in Yemen (Hopkins, 2006) . The Arabia coffee was originated from Yemen where the seeds are roasted to perfection and brewed. The same process takes place in the future as well nothing changed. Coffee got famous and started to spread out from the Middle East to Venice and North Africa where traders of old would brought coffee form the Middle Eastern sides to those shores for trade and it expanded far beyond that of Venice to the far reaches of Europe itself and then moving it way all around the world (Villaneuva *et al*., 2006).

Coffee is categorized under the class Magnoliopsida, the order of Gentianales. They are in the Rubiaceae family, of which it is sub into the family of Ixoroideae and originated from the genus Coffea (Allison and Mellissa, 2013). They are considered shrubs which usually grow less than 5 meter, leaves dark green with a glossy touch, wide, different layers and a bit oval in shape (Allison and Mellissa, 2013). The petioles fuse at the base which forms an interpetiolar stipules. Their flower are clusters in white with a fragrant odor. The berries

colour changes from green when immature, yellow to crimson when ripe and black when drying. Most of the berries usually contains two seed rarely one seed (Allison and Mellissa, 2013). Two most notable and prized coffee around the world is *coffea arabica* and *coffea canephora* which make up most of the coffee trade markets. The coffee produce are usually from the seed of the berries which they extract and roast it to a degree before brewing. Coffee plant prefer a climate which is humid and hot all year round and this makes the countries around the equatorial belt favorable for these plants.

The process of coffee berries before being made into the coffee is an exciting one. It all starts from being handpicked or harvested overall by a machine. They then undergo either a drying process or a less hassle method which is known as the wet strip pick. The coffee still needs to be dried to expel all the humidity. After drying process has been completed they undergo roasting, where the dried coffee beans called green beans. They are subjugated to a temperature of 200 ºC when roasting is taking place (Cornelis and El-Sohemy, 2007). During this intense roasting process the sucrose in the beans are rapidly lost which gives rise to the brown caramel looking colour of the coffee. Next comes the brewing where the coffee has to be grounded and mixed with hot water long enough for the beans to give out the flavor enclosed in the beans. The ideal temperatures for the beans to be fully optimized are within the range of 90 ºC to 100 ºC. There are many methods in brewing which includes boiled, steeped or pressurized. Cold method of brewing coffee is also eligible but the taste and acidity differs from those of the other 3 methods mentioned and true coffee enthusiast prefer hot over cold.

This century we have seen an increase in the coffee industry, where more types of bizarre ways of coffee being made and produced such as the Nespresso produced by Nestle which

the coffee is grounded in a capsule and by using the Nespresso machine it can produce coffee in a unique way which taste even better and yields more coffee than normal grounded coffee. The cost on the other hand is expensive so that is why Nespresso has not been a phenomenal thing as a capsule is very costly. Other than that, adulterated coffee mixed are being an issue of concern now as many of these coffee are being spiked with drugs ranging from types of sexual enhancers to those of herbal beauty products. Many of this coffee can be found mainly on the South East Asia side and it is widely used in Thailand, Malaysia and Indonesia.

An adulterant is a substance found inside different substances for instance nourishment refreshments and ointment, in spite of the fact that not took into account lawful or different reasons (Coley, 2005). The expansion of adulterants is called adulteration. An adulterant is different from, for instance, allowed sustenance added substances. There can be a scarce difference in the middle of adulterant and added substance; chicory may be added to coffee to decrease the expense, this is adulteration if not announced, however may be tested on the label (Coley, 2005). The term tainting can be utilized as this sorts of items are defiled with abundance drugs over the safety level. Adulterants added to decrease the measure of extravagant item in illegal medications are called cutting executors. Intentional expansion of lethal adulterants to sustenance or different items for human utilization is dangerous (Coley, 2005).

Eurycoma longifolia aslo known as "Tongkat Ali" from the family Simaroubaceae is a flowering plant, found in South East Asia only from the extent of Loas till Indonesia. It is found in abundance in Malaysia and is one of the famous plants for its medicinal purposes especially in the boostage of a man's libido (Abdullah *et al*., 2004). In Malaysia the usage

of E. longifolia are usually found in beverages especially coffee called "Power Root". Since many herbs have similar effect and the rarity of *E. loangifolia*, many products claim to have its properties are sold throughout the market (Abdullah *et al*., 2004) . Now days many have claimed that Tongkat Ali is being abused with other types of synthetic drugs which are added into these coffees and claimed to be Tongkat Ali. Many have reported being ill and having side effects by consuming this types of coffee. Due to its lower cost compared to the original Tongkat Ali it is popular among the people in the rural areas.

Abisia pumila also known as kacip fatimah, from the family Primulaceae which originated from Malaysia (Wan Hassan, 2006).They are herbaceous plant and grow under shades of the rainforest. *Labisia Pumila var alata*, *L. pumila var pumila*, and *L.pumila var Ianceolata* are the 3 species found to be thriving in Malaysia (Wan Hassan, 2006). Each has a different usage and each usage has their own personal beneficial properties and it is advisable each is used to its full potential at the right way. The plant is believed by the locals to give boost to a woman's inner and outer beauty (Wan Hassan, 2006). Before utilization back in the days, the whole plant will be boiled for the extract which is consumed or used during bathing. Traditional usage of the plant for centuries in the Malay cultures had been used by a variety of women for after birth treatment and menstrual pain relief of irregularity (Kuah Guan Oo, 2014). Now they are made into tablets and it is closely related to Tongkat Ali.

Ginseng is any of 11 types of moderate developing lasting plants with perennial roots. They thrive in cooler atmosphere (Baeg *et al*., 2013). *Panax vietnamensis*, found in Vietnam, is the southernmost ginseng known. Ginseng is portrayed by the vicinity of ginsenosides (Baeg *et al*., 2013). Siberian ginseng *Eleutherococcus senticosus* is in the same family, however not variety, as genuine ginseng. Like ginseng, it is thought to be an adaptogenic

herb (Baeg *et al*., 2013).. The dynamic mixes in Siberian ginseng are eleutherosides, not ginsenosides. Rather than a perennial root, Siberian ginseng has a woody root. Only a certain amount but it's getting lesser and lesser that ginseng is used in coffee these days as they are very expensive and hard to come by. Other than that some ginsengs are said to give a bit of a side effect such as nosebleed, diarrhea, nuasea, headache, high and low blood pressure and breast pain (Keifer and Pantuso, 2003). White blood cells, T cells as well as cytotixc T cells benefits greatly if daily intake of the root is practised (Keifer and Pantuso, 2003).

2.2 Sildenafil

Sildenafil also known as the famous 'Viagra' is mainly used in the treatment of erectile dysfunction and pulmonary arterial hypertension (Vardi and Nini, 2007). Diabetes mellitus patient are often being prescribe sildenafil as a primary source of erectile dysfunction (Vardi and Nini, 2007). Sildenafil citrate on the other hand is also used in the treatment of the rare disease of pulmonary hypertension which relaxes the arterial wall leading to the low pressure resistance of the wall. This may lead to heart failure but sildenafil helps by acting on the walls of the smooth muscle of not only the penis but the arterial wall as well without causing any vasodilation (Bolell *et al*., 1996). Sadly, sildenafil have been said to have many side effects including headache, heartburn and flushed skin. Some have been known to have prolonged erection which last for hours and leads to the damage of the penis and some have been reported to have hearing loss and a weak heart once the effect wears off. Sildenafil also disrupts patient undergoing HIV treatment with protease inhibitor as it prolongs the plasma half-life (Vardi and Nini, 2007). Sildenafil can be detected in the body

if any fatal overdose case ever happens by assessing the whole blood, serum or plasma of the victim by the pharmocokinetic status.

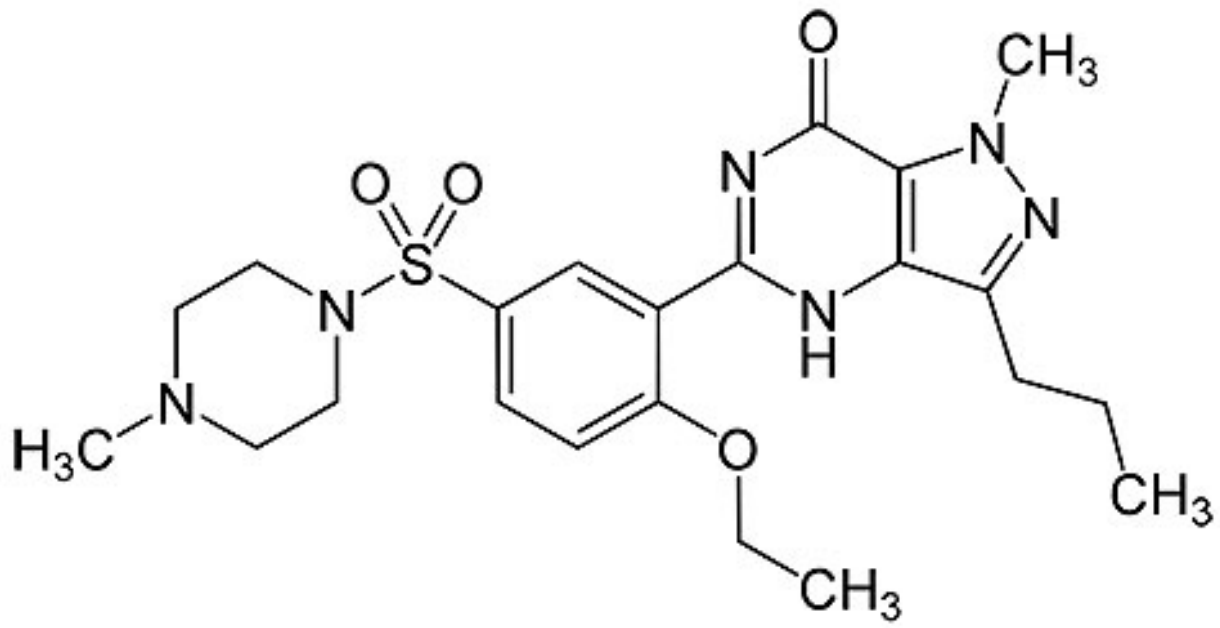


Figure 2.1: Sildenafil molecular structure (Vardi and Nini, 2007).

The system of activity of this drug includes the insurance of cyclic guanosine monophosphate (cGMP) from debasement by cGMP-specific phosphodiesterase sort 5 (PDE5) in the corpus cavernosum (Vardi and Nini, 2007). Nitric oxide (NO) in the corpus cavernosum of the penis ties to guanylate cyclase receptors, which brings about expanded levels of cGMP, prompting smooth muscle vasodilation of the intimal pads of the helicine veins. This lead to unwinding prompts relaxation thus expanded flow of red fluids into the supple parts, bringing on an erection (Vardi and Nini, 2007). Sildenafil is an intense and particular inhibitor of cGMP- PDE5, which is in charge of debasement of cGMP in the corpus cavernosum (Vardi and Nini, 2007). The molecular structure of sildenafil is like that of cGMP and goes about as an aggressive tying specialist of PDE5 in the corpus cavernosum, bringing about more cGMP and better erections. Without sexual incitement,

and in this manner absence of actuation of the NO/cGMP framework, sildenafil ought not bring about an erection. Different medications that work by the same system include those of tadalafil and vardenafil. Sildenafil is metabolise in the liver by hepatic digestion system utilizing cytochrome p450 3A4 (CYP3A4) chemicals, primarily CYP3A4, additionally by CYP2C9 minor course hepatic isoenzymes. The real result of metabolisation by these catalysts is N-desmethylated sildenafil, which is metabolized further. This metabolite additionally has a natural affinity for the PDE receptors, around 40% of that of sildenafil. Consequently, the metabolite is in charge of around 20% of sildenafil's activity (Web *et al*., 1999) . Sildenafil is discharged as metabolites overwhelmingly in the excrement and to a lesser degree in the urine. In the event that brought with a high-fat feast, ingestion is decreased; the time taken to achieve the most extreme plasma focus increments by around 60 minutes, and the greatest fixation itself is diminished by about one-third (Webb *et al*., 1999).

2.3 Tadalafil

Tadalafil as its counterpart sildenafil and vardenafil and works the same in treating erectile dysfunction and the treatment of pulmonary arterial hypertension. Besides that it also treats the sign and symptoms of benign prostatic hyperplasia (BPH) (Bischoff, 2004). Tadalafil has been utilized as a part of pretty nearly 15,000 men taking an interest in clinical trials, and more than eight million men overall. The most well-known reactions when utilizing tadalafil are cerebral pain, stomach distress or agony, acid reflux, burping, heartburn, back pain, muscle throbs, flushing, and stuffy or runny nose. These symptoms mirror the capacity of PDE5 hindrance to bring about vasodilation, and ordinarily go away following a couple of hours. Back pain and muscle aches can happen 12 to 24 hours in the wake of

taking the medication, and the manifestation of the side effect diminishes in the following 48 hours (Bischoff, 2004). Nonarteritic anterior ischemic optic neuropathy (NAION) vision impairment have been reported to occur in certain patients.

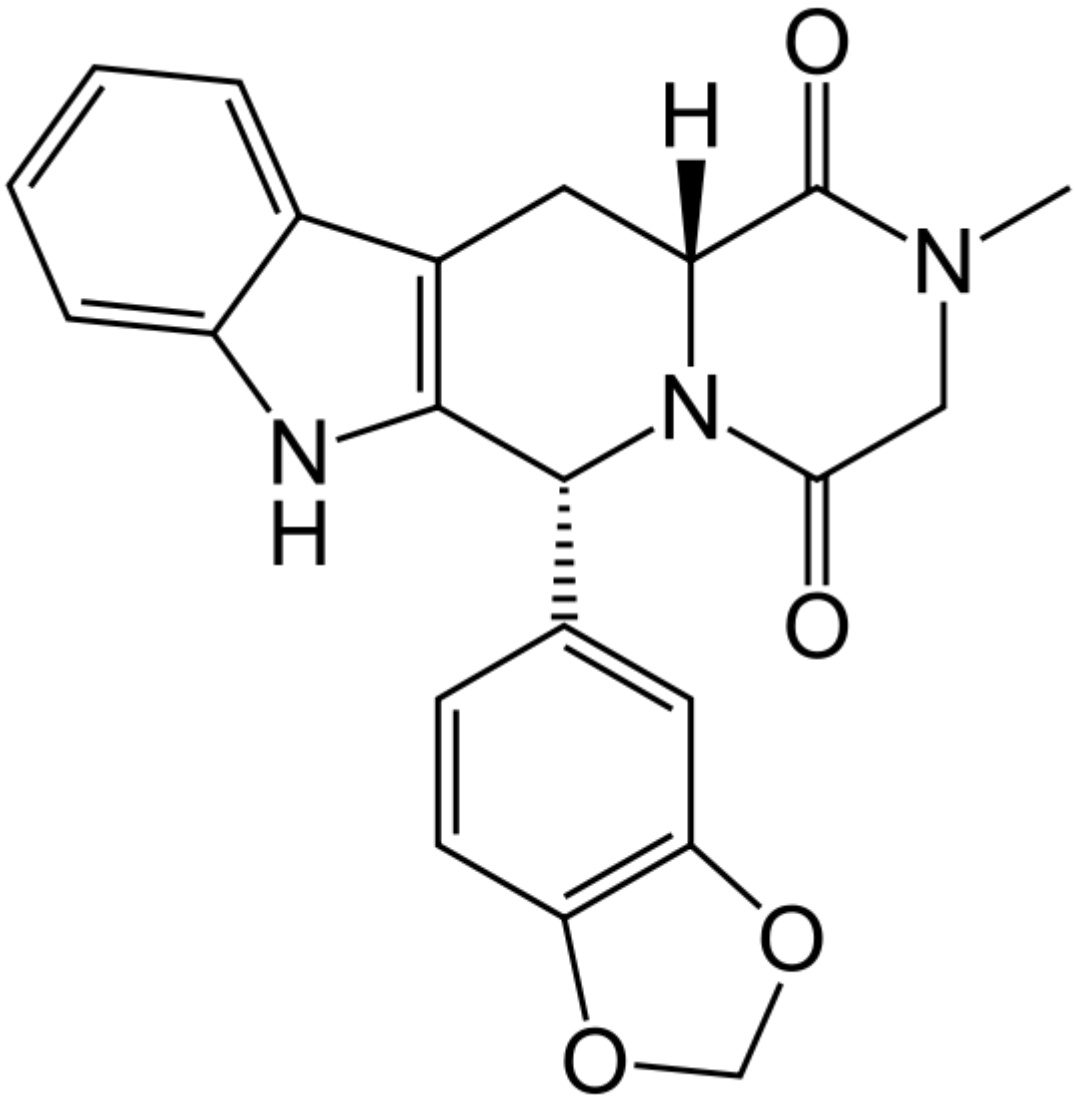


Figure 2.2: Tadalafil molecular structure (Bischoff, 2004).

Since PDE5 inhibitors, for example, tadalafil may bring about briefly low pulse; natural nitrates ought not to be taken for no less than 48 hours subsequent to taking the last

measurement of tadalafil (Bischoff, 2004). Utilizing natural nitrites inside of this timeline may expand the danger of life-debilitating hypotension. Since individuals who have taken tadalafil inside of the previous 48 hours can't take natural nitrates to calm angina, these patients has to look for prompt restorative consideration in the event that they encounter anginal chest pain (Daugan *et al*., 2003). In the occasion of a therapeutic crisis, paramedics and medicinal work force are advised to be told forth of any late dosages of tadalafil. Tadalafil is metabolized directly by the hepatic CYP3A4 catalyst framework (Daugan *et al*., 2003). The vicinity of different medications which effect this framework can shorten tadalafil half-life and decrease serum levels, and henceforth adequacy, of the medication.

Tadalafil longer half life compared to vardenafil and sildenafil which results in more lasting hours of action. This long half-life leads to the usage of tadalafil as a good source of medicine for the treatment of heart disease. Erection of the penis occurs by the blood flow from the relaxation of the smooth muscle. This helps in the release of nitric oxide which promotes the cyclic guanosine monophosphate (GMP/cGMP) in the penile smooth muscle cells (Daugan *et al*., 2003). Erectile function are enhanced by increasing the amount of cGMP but at the same time the inhibition of PDE5 and sexual stimulation is a must for the nitric oxide to be released or else tadalafil would not take effect (Daugan *et al*., 2003).

2.4 Vardenafil

Same as its counterpart, vardenafil is similar in function in inhibiting the PDE5 enzyme for the usage of erectile dysfunction patients. Trade names for vardenafil are "Levitra", "Staxyn", and "Vivanza". Tadalafil, sildenafil, and vardenafil all demonstrate by hindering the PDE5 enzyme. These medications additionally hinder other chemicals. Both sildenafil and vardenafil are effective even more than tadalafil in hindering PDE6 as well as PDE

1(Bischoff, 2004).Some reported to be more sensitive to light and to be able to see a bluish tint. PDE1 is found in the cerebrum and parts of the cardiovascular system (Bischoff, 2004). Positioning of the nitrogen molecule at the piperazine methly ring is what contradicts vardenafil and sildenafil citrate. Tadalafil is usually exclusive in regards to both sildenafil and vardenafil. Vardenafil's reasonably brief highly effective time is just like however pretty more than sildenafil's. Beyond its information for sex ineffectiveness, vardenafil may be effective in the therapy of beginning ejaculation, where it might basically develop time from transmitting to ejaculation (Bischoff, 2004).

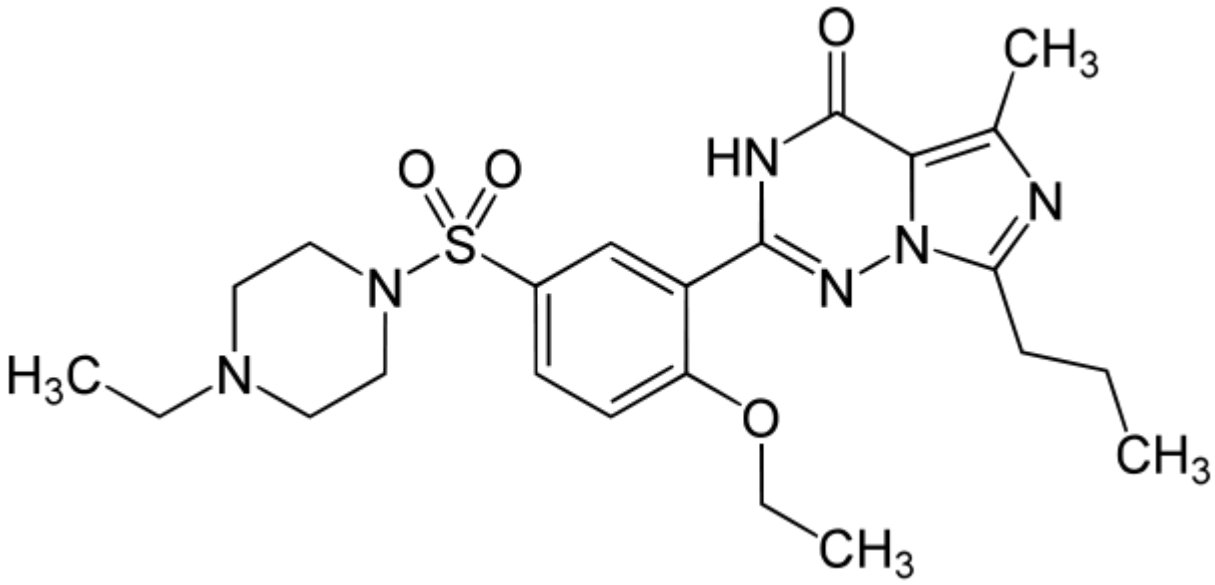


Figure 2.3: Vardenafil molecular structure (Bischoff, 2004).

Nausea, abdominal pain, back pain, photosensitivity, abnormal vision, eye pain, facial edema, hypotension, palpitation, tachycardia, arthralgia, myalgia, rash, itch and priapism are the side adverse effect of common vardenafil. Same as the other counterparts this drug cannot be consumed with nitrate based medication as it might lead to the invocation of life threatening hypotension (Daugan *et al*., 2003).

2.5 UV-Visible spectroscopy

It is the reflecting and absorbing action on a unseen plane the ultraviolet-visible (UV) which means light in the range of UV and near infrared ranges which is visible and adjacent ranges (Tatzer *et al*., 2005). The chemicals involved undergo electronic transition in the region of spectrum which mimics the technique of the fluorescence spectroscopy but rather than the excited state to the ground state it is actually the opposite from the ground state to the excited state (Skoog *et al*., 2007).

Ion of metal transition are able to be shaded which must absorbs visible light on the grounds that d electrons inside of the metal molecules can be energized starting with one electronic state then onto the next. The shading of metal particle arrangements is firmly influenced by the vicinity of different species, for example, certain anions or ligands (Boggia *et al.,* 2013) . This means, maximum absorption (λmax) can be obtained when adding another component to increase the shade and wavelength (Skoog *et al*., 2007). Whereas for organic compounds, assimilation with high level of light with conjugation on the range. Solvent plays a role in the factor of compounds which are water soluble or only soluble in certain compounded solution as they are greater when exposed to UV absorbance but not all are usable only a certain few. The absorbance spectrum of an organic compound is affected by the pH and polarity will as well (Ozaki *et al.*, 2007). The solution will always be directly proportional to concentration of the absorbance of the solution and the length of path by abiding the Beer-Lambert law (Skoog *et al*., 2007) hence the machine identifies the concentration of absorbance in the particular solution. Besides that the machine method can be used in the determination of the kinetics rate constant of that to the chemical reaction. During the reaction in the solution the colour present must shift from reactants to the

product in order for the UV-Visible spectroscopy to be viable for this method. Other than that, rate constants of a particular reaction can be obtained by this method at specific time intervals giving the example of mercury dithizonate, the solution can be turned blue when a light is shine to it and after that run the UV-Visible test to observe the change state of the solution from blue to yellow solution over the interval of 10 sec to see how the absorbance work (Sertova, 2000).

2.6 Chemometrics

Chemometrics is the best approach in the collectivity of data by technological means which acts as an interdisciplinary method using methods employed in core data analysis. This allows the better understanding of the chemical behavior through research using mathematical science data (Manley and Geladi, 2011). Both physical and chemical data were obtained in the spectra of the UV-Visible spectra (Ozaki *et al.*, 2007). Chemometrics is connected to tackle both expressive and prescient issues in chemistry. This allows better understanding of the fundamentals of chemistry and mathematics in a framework taking into account the fundamental elements necessary for both of this to intertwine. New properties and interest can be expected with the application of this system as a whole (Ozaki *et al.*, 2007). In both cases, the datasets can be little yet are regularly vast and very mind boggling, including hundreds to a large number of variations, and unique vast observations. It intensely utilized as a part of analytical chemistry and metabolomics, and the advancement of enhanced chemometric method for analysis additionally keeps on propelling the cutting edge in systematic instrumentation and technique (Ozaki *et al.*, 2007). It is an application driven request, and thus while the standard chemometric methodology are by and large used.

Principal component analysis (PCA) is an unsupervised clustering data technique used in visualization and pattern recognition (Cozzolino *et al*., 2009). It reduces the multivariate sets of data dimensionally. New variables which are the principal components (PC) are the original variables which are linear functions and each consecutive PC acts at a random notion since they are in perpendicular to another (Manley and Geladi, 2011). Every PC consists of scores and loading counterpart, where it indicates the relation between samples and the loadings shows how these variables relate to one another. Each PC loading are shown as a line plot and the scores as the scatter plot and the successful interpretation of measured spectroscopic data.

At the point when exceedingly collinear variables exist in an information set frequently utilizing another direction framework permits better perception of the accessible data (Geladi *et al*., 2004; Lavine & Workman, 2005). Mean-focusing can likewise be connected to the information network, to guarantee ideal depiction of the investigated specimens by new PCs. With mean-focusing the normal of the information network is subtracted from every individual example. PCA is a bilinear displaying procedure, in this way before PCA can be used to multivariate images such as the 3D square of information modelling the hypercube must be developed into a two dimensional information set or table (Geladi *et al*., 2007). A multivariate picture comprises of three dimensions, where the initial two are spatial (x,y) and the third is a spectral measurement (λ).

Partial least squares regression (PLS) is closely related to component regression but is a new statistical method which is in relation with one another. Instead of hyperplanes of lowest difference between the reaction and separate factors, a straight line regression design is acquired by predicting the expected factors and the visible factors to a new area (Amigo

et al., 2009).They are known as bilinear factor models where the both axis lies onto new planes. Partial least squares Discriminant Analysis (PLS-DA) is a variable used when the Y is explicit (Amigo *et al.*, 2009). PLS-DA creation came to be when a conventional PLS model was built on the variable group indicator. This is further explained by predicting that the X spectral space data for each new sample and by identifying how the predicted data belonged to the class Y (Liu *et al*., 2008).

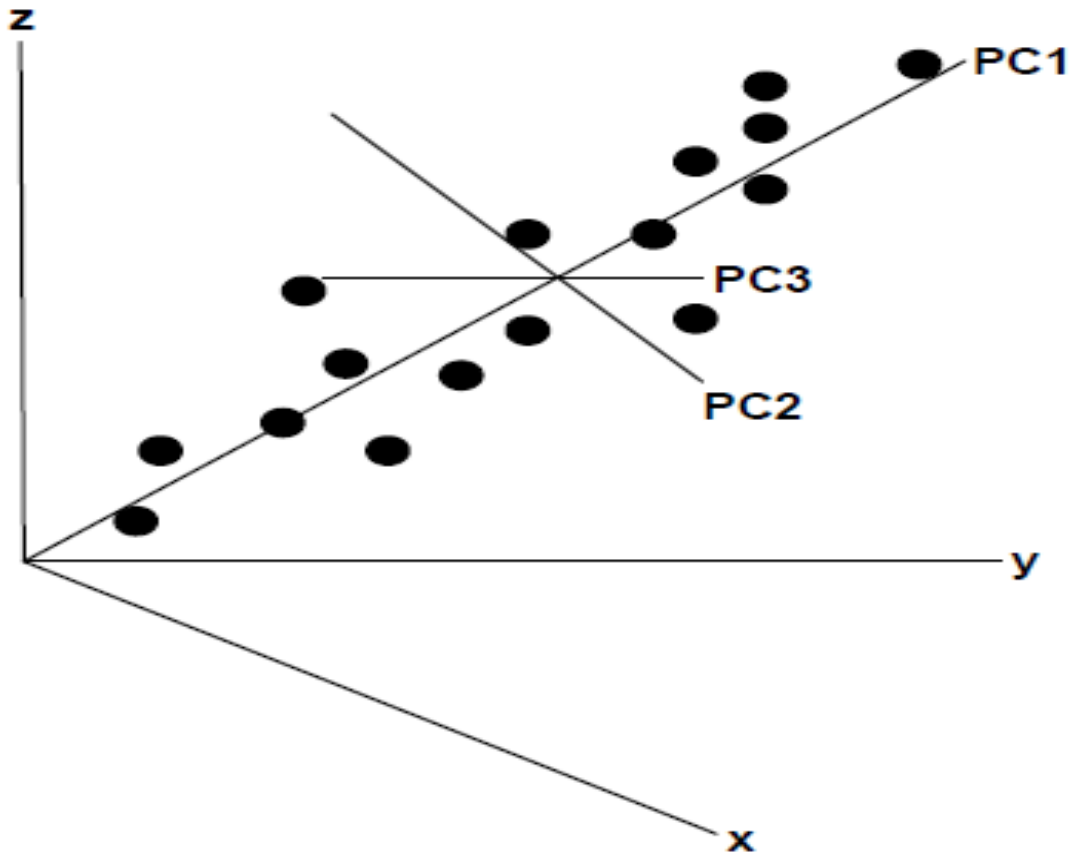


Figure 2.4: Coordinates formed by the PCA, and samples may be defined by the PC 1, PC 2 or PC 3 besides the x, y and z normally used. PC 3 is considered a noise in the data.

CHAPTER 3

METHODOLOGY

3.1 Materials and method

Seven products of premixed coffee were chosen from various brands with three claimed to be adulterated and the other four to be normal coffee and two out of the five were claimed to be containing special herbal properties. Each material was purchased from various shops located all around Malaysia. Absolute ethanol (VMR Chemicals, France) was used as the solvent to dissolve the coffee premix.

3.2 Sample Preparation

All the premixed coffees were in the powdered form; therefore extraction process is not required. The premixed coffee was readily dissolved in ethanol and the suspended particles were filtered. They were mixed in a centrifuge tube (2ml) and shaken steadily for a minute. The concentration of sample was 3mg of coffee in 1 ml of ethanol. 30 replicates of each premixed coffee were taken from each brand totaling to 210 samples.

3.3 UV-Vis

The method used for the experiment was performed using a UV-Visible Spectroscopy (Perkin Elmer, Waltham, Massachusetts, United States of America) that comes with software included (Lambda 25) for the range of 0 nm - 600 nm with 1 nm resolution. The cells used were rectangular cuvette measuring 2mm (UVette ® 220-1600nm disposable single sealed cuvettes, Eppendorf, Germany). Absolute ethanol was used as blank.

3.4 Multivariate Data Analysis

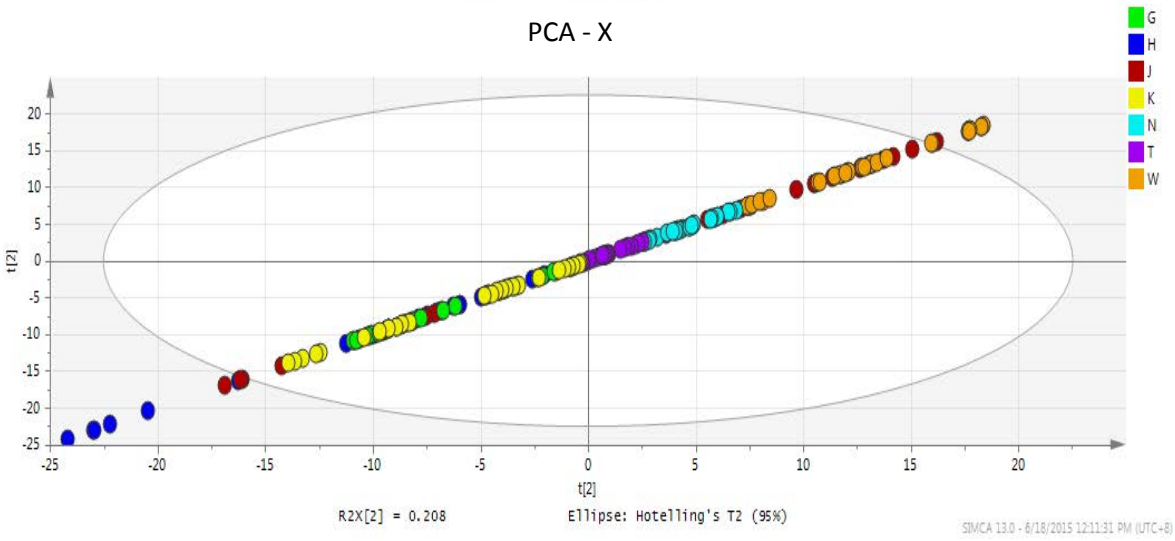
All the spectra were baseline corrected and smoothed before conversion to ASCII format and then converted into a single table of Microsoft Excel (2007-2013). The processed data were then imported into SIMCA-P version 13.0 (Umetrics, Umeå, Sweden) for multivariate statistical analysis in a scaling parameter of unit variance (UV). PCA was adopted for the initial exploratory data analysis whereby the scores plot reflected separation among the samples. However, orthogonal partial least squares discriminant analysis (OPLS-DA) and PLS-DA was preferably employed due to the clearly observed discrimination of the samples in accordance with their contents or adulterants. The employed model was described by the criteria of R^2, which indicated the goodness of fit, and Q^2, the goodness of prediction(Yusof et al., 2014). The variables influences on projection (VIP) values exceeding 1.0 were selected and not removed as when removing any values exceeding 1.0 will alter the plot of each scatter plot (Yusof et al., 2014). Moreover, the significance of each model established was validated by P value ($P < 0.05$) of CV-ANOVA as the supporting information.

CHAPTER 4

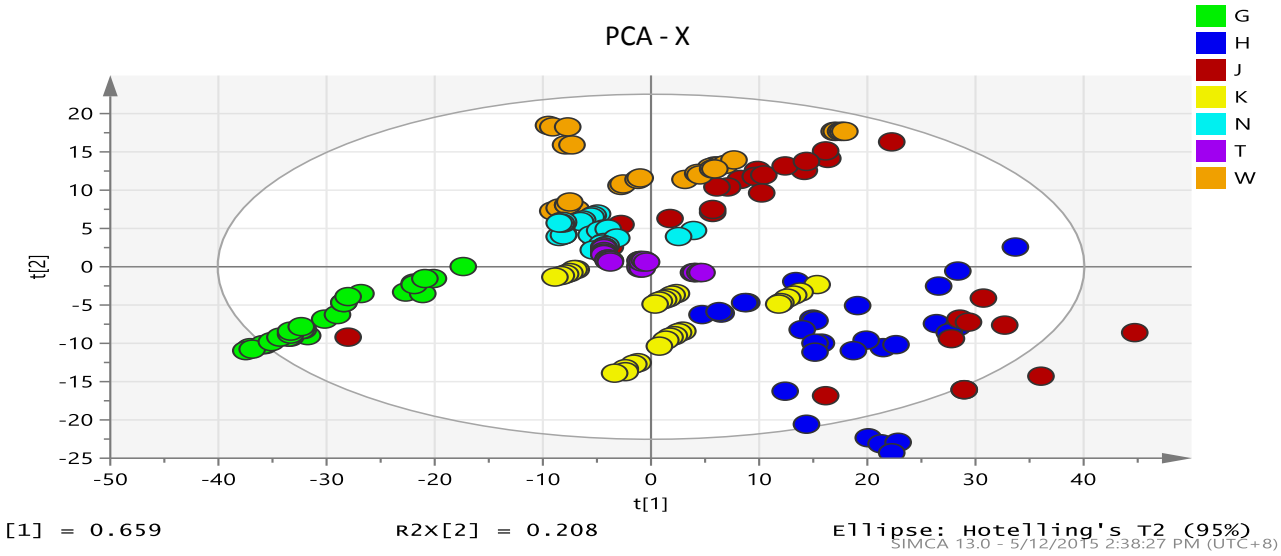
RESULTS & DISCUSSION

The figures below represent the same scatter score plot for a PCA model but differs as the X axis was changed to consider which scatter score plot gives out the best clustering and it is seen by that, Figure 4.1 b) is the most acceptable clustering model as we can see below the groups has the lowest discriminant. Figure 4.1 a) shows a straight line of the group clustering indicating the score plot X and Y axis correlates with each other. From this given types of model the most acceptable is Figure 4.1 b) as it is the best chosen by the program after processing the data as the clustering of each of the data on the scatter score plot are clustered within the vicinity of their group, with the exception of some outliers. Therefore the chosen model for PCA, OPLS-DA and PLS-DA subplot.

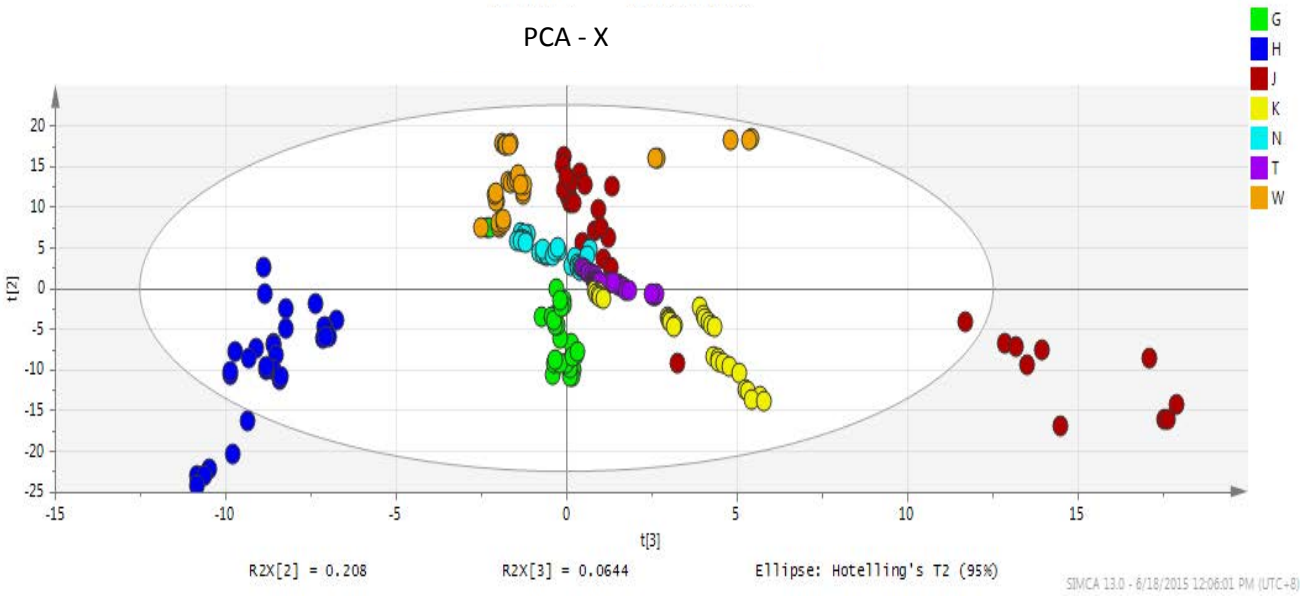
a)



b)


PCA - X
G
H
J
K
N
T
W
t[2]
t[1]
[1] = 0.659
R2X[2] = 0.208
Ellipse: Hotelling's T2 (95%)
SIMCA 13.0 - 5/12/2015 2:38:27 PM (UTC+8)

c)


PCA - X
G
H
J
K
N
T
W
t[2]
t[3]
R2X[2] = 0.208
R2X[3] = 0.0644
Ellipse: Hotelling's T2 (95%)
SIMCA 13.0 - 6/18/2015 12:06:01 PM (UTC+8)

d)

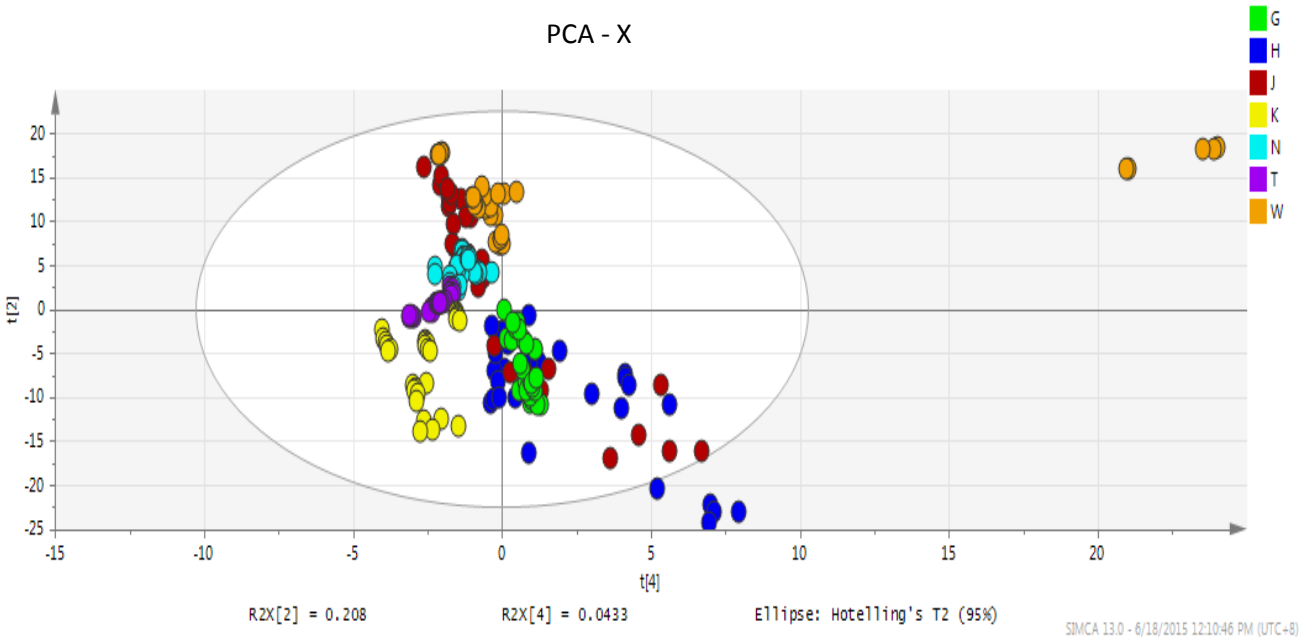


Figure 4.1: 4 PCA score scatter plot models with different types of X axis ranging from a) t[2] , b) t[1] , c) t [3] , d) t[4].

4.1 PCA Model

4.1.1 Summary of Fit

R^2 or also known as fit estimates the goodness of fit and it explains the variation of the data. As shown in the figure below, R^2 is the percentage of set for PCA as well as how well the data accepts the model. A good model have a R^2 of nearly to 1 but it still does not suffice as is having unpredictable models which are poor models with a large R^2 still generates much noise (Manley and Geladi, 2011).

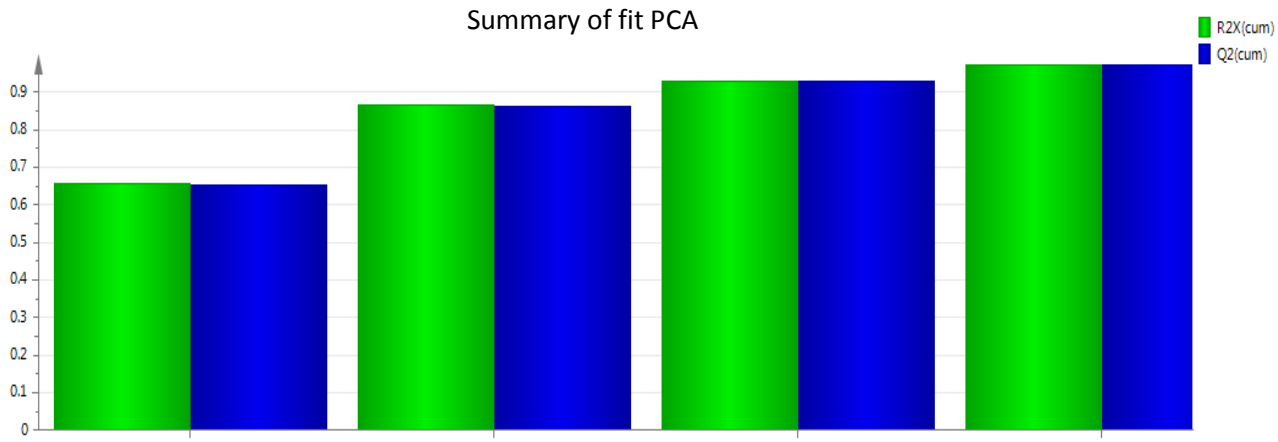


Figure 4.2: The summary of fit for PCA where Q^2 does not exceed R^2.

Q^2 is known as predictive ability which estimates the goodness of prediction by cross validation. Q^2 is the percentage of variation determined by the cross validation for PCA. It dictates on how well the information is predicted. A large Q^2 ($Q^2 > 0.5$) indicates good predictivity. When noise is present, an unquality Q^2 is obtained, or when the model is dominated by few scattered outliers (Manley and Geladi, 2011).